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PATENT SPECIFICATION

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Date of Application and filing Complete Specification: Oct. 22, 1963.
No. 41675/63.
Application made in United States of America (No. 232260) on Oct. 22, 1962
Application made in United States of America (No. 232872) on Oct. 25, 1962
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Index at acceptance:—C3 H3

ERRATUM

SPECIFICATION No. 1,001,173
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Page 1, Heading, Application made in U.S.A. No. 232,872, *for* "Oct. 25, 1962" *read* "Oct. 24, 1962"

THE PATENT OFFICE
16th February 1966

This invention relates to the production of galactose oxidase.

Galactose oxidase is an enzyme which catalyses the oxidation of galactose in the presence of molecular oxygen. The activity of this particular enzyme is outstanding with respect to its specificity for a galactose substrate. Its activity upon glucose and simple sugars other than galactose is practically nil. Galactose oxidase is useful in detecting galactose when used with suitable indicator systems in diagnostic compositions, for converting galactose to its oxidation products, which is of utility in freeing certain foods of galactose, and in other industrial applications such as the removal of oxygen from various materials.

This enzyme has been produced in the past by the fermentation of media containing galactose and a plurality of mineral constituents.

These previous methods, however, have in general produced a slow growth of the enzyme-producing organism and have resulted in relatively poor yields of enzyme.

The present invention provides a process for the production of galactose oxidase which comprises growing a culture of *Polyporus circinatus Fr.* in an aqueous fermentation medium containing up to 2.0% (w/v) of a carbohydrate source comprising one or more of galactose, lactose, sucrose, raffinose, glucose, fructose, mannose, sorbose, beet pulp, orange pulp, flour, starch, mannitol, sorbitol, inositol and glycerol, from 0.01%

[*Price 4s. 6d.*]

ammonium acetate, formamide and urea. Also included in the medium are certain conventional nutrients which have been found to facilitate the growth of the organism.

The medium may also contain a lipid material.

In producing galactose oxdiase according to the method of this invention the cultures of *Polyporus circinatus Fr.* are allowed to stand in flasks containing the culture medium to initiate growth. After standing for 2 to 3 days the culture is sufficiently developed to be transferred to a fermentation medium at a pH of from 6.3 to 6.8 for the production fermentation. During the fermentation, the inoculated medium is agitated and maintained at or near room temperature. For example, temperatures in the range of about from 23°C. to 30°C. are satisfactory. Air is passed through the medium in order to insure thorough mixing and suitable aeration.

The fermentation may be followed by a suitable assay technique to determine when the peak yield of galactose oxidase has been reached. It has been found that this yield is reached within a period of about from 72 to 120 hours following inoculation of the fermentation medium.

Upon reaching the desired enzyme yield, the mycelium is removed by filtration and the filtered beer processed to recover the galactose oxidase as will be hereinafter described.

The carbohydrate source of the fermentation medium comprises monosaccharides and

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COMPLETE SPECIFICATION

Process for the production of Galactose Oxidase

We, MILES LABORATORIES, INC., a corporation organised and existing under the laws of the State of Indiana, United States of America, of 1127 Myrtle Street, Elkhart, State of Indiana, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

This invention relates to the production of galactose oxidase.

Galactose oxidase is an enzyme which catalyses the oxidation of galactose in the presence of molecular oxygen. The activity of this particular enzyme is outstanding with respect to its specificity for a galactose substrate. Its activity upon glucose and simple sugars other than galactose is practically nil. Galactose oxidase is useful in detecting galactose when used with suitable indicator systems in diagnostic compositions, for converting galactose to its oxidation products, which is of utility in freeing certain foods of galactose, and in other industrial applications such as the removal of oxygen from various materials.

This enzyme has been produced in the past by the fermentation of media containing galactose and a plurality of mineral constituents.

These previous methods, however, have in general produced a slow growth of the enzyme-producing organism and have resulted in relatively poor yields of enzyme.

The present invention provides a process for the production of galactose oxidase which comprises growing a culture of *Polyporus circinatus Fr.* in an aqueous fermentation medium containing up to 2.0% (w/v) of a carbohydrate source comprising one or more of galactose, lactose, sucrose, raffinose, glucose, fructose, mannose, sorbose, beet pulp, orange pulp, flour, starch, mannitol, sorbitol, inositol and glycerol, from 0.01% to 0.15% (w/v) of nitrogen in the form of a primary organic nitrogen source comprising one or more of casein, casein hydrolysate, whole milk powder, meat extracts, fish solubles, autoclaved yeast, soybean protein hydrolysate, cottonseed meal and stick liquors, and from 0.005% to 0.1% (w/v) of nitrogen in the form of an auxiliary nitrogen source comprising one or more of glycine, aspartic acid, asparagine, glutamic acid, alanine, ammonium acetate, formamide and urea. Also included in the medium are certain conventional nutrients which have been found to facilitate the growth of the organism.

The medium may also contain a lipid material.

In producing galactose oxdiase according to the method of this invention the cultures of *Polyporus circinatus Fr.* are allowed to stand in flasks containing the culture medium to initiate growth. After standing for 2 to 3 days the culture is sufficiently developed to be transferred to a fermentation medium at a pH of from 6.3 to 6.8 for the production fermentation. During the fermentation, the inoculated medium is agitated and maintained at or near room temperature. For example, temperatures in the range of about from 23°C. to 30°C. are satisfactory. Air is passed through the medium in order to insure thorough mixing and suitable aeration.

The fermentation may be followed by a suitable assay technique to determine when the peak yield of galactose oxidase has been reached. It has been found that this yield is reached within a period of about from 72 to 120 hours following inoculation of the fermentation medium.

Upon reaching the desired enzyme yield, the mycelium is removed by filtration and the filtered beer processed to recover the galactose oxidase as will be hereinafter described.

The carbohydrate source of the fermentation medium comprises monosaccharides and

polysaccharides which are capable of being utilised as a nutrient source for the growth of fungi, and includes both carbohydrates and reduced carbohydrates. For example, galactose is a very satisfactory carbohydrate source for the fermentation medium. The other carbohydrates which may be used are lactose, sucrose, raffinose, glucose, fructose, mannose, sorbose, beet pulp, orange pulp and various flours and starches. The reduced carbohydrates which have been found effective for this purpose are mannitol, sorbitol, inositol and glycerol. The carbohydrate may be utilized in the fermentation medium in any concentration up to 2.0% (w/v).

The primary organic nitrogen source, which forms another essential element of the fermentation medium in the process of this invention, is a material which in general is a protein or a hydrolyzed protein. A wide variety of nitrogen-containing materials is utilised for this purpose: casein, casein hydrolysate, whole milk powder, meat extracts of various types, fish solubles, autoclaved yeast, soyabean protein hydrolyzate, cotton-seed meal and various stick liquors. Other proteins or protein hydrolyzates may also be utilised if desired. These products may serve as carbon and vitamin sources as well as being the primary nitrogen source. The primary source of organic nitrogen is used in the fermentation medium in a concentration of from 0.01% to 0.15% nitrogen (w/v).

The use of the additional or auxiliary nitrogen source referred to above greatly increases the yields of galactose oxidase and substantially reduces the time taken for the fermentation cycles. This auxiliary nitrogen source comprises amino acids, such as glycine, aspartic acid, asparagine, glutamic acid and alanine; ammonium salts of carboxylic acids, such as ammonium acetate, and various other organic nitrogen compounds, such as formamide or urea, may alternatively be used. The auxiliary nitrogen source is used in the fermentation medium in a concentration equivalent to from 0.005 to 0.1% nitrogen (w/v).

The addition of a lipid material to the fermentation medium has also been found greatly to enhance the growth of the galactose oxidase producing organism and to produce substantially greater yields of galactose oxidase in shorter fermentation cycles. The lipid materials that may be utilized for this purpose include fats, oils and waxes. Examples of such materials are mutton tallow, lard oil, raw linseed oil, peanut oil, olive oil, soybean oil, vegetable oil, corn oil and cotton-seed oil. In addition, other materials which may be used and which it is intended to include within the terminology "lipid materials" are long chain fatty alcohols such as octadecanol, long chain fatty acids such as stearic acid, and industrial fat-like esters such as sorbitan trioleate. The lipid material may be used in the fermentation medium in a concentration of from 0.5% to 2.0% (w/v).

In addition to the above described ingredients of the fermentation medium which form the distinguishing features of this invention, the use of other commonly used ingredients is also desirable. For example, mineral salts, such as magnesium sulphate, inorganic nitrogen compounds, such as ammonium nitrate and vitamin mixtures may be deemed desirable.

After the fermentation has proceeded to the desired extent and the yield of galactose oxidase as above mentioned has attained a maximum or least a commercially desirable level, the fermentation is stopped and the fermentation beer harvested. For recovery of the enzyme a preferred method is precipitation by adding various water-miscible organic solvents. For example, acetone, ethanol or isopropanol precipitation may be used for this purpose.

Before harvesting the enzyme, the fermentation beer is filtered, cooled to 15-10°C. with ice bags, fortified with 5-10 grams of potassium dihydrogen phosphate per litre and adjusted to pH 6.3 to 6.8. The addition of the phosphate helps to lower the pH of the beer from about 8 to the desired range. It also helps to increase the salt content of the beer thus facilitating the precipitation of the galactose oxidase. The beer is then added slowly with stirring in two volumes of cold acetone. (Cooled to about —20°C. with solid carbon dioxide.)

In some instances it has been found useful to also add a salt commonly used for "salting out" enzymes from aqueous solution, such as ammonium sulphate. Where ammonium sulphate is used it may be added (as a saturated solution) in a quantity of about 5-10 millilitres per litre of original beer. The acetone-beer mixture is then allowed to stand for a period of about two hours during which time the enzyme settles out. Thereupon the supernatant is decanted and the precipitated enzyme collected, washed several times with dry acetone and dried. If desired the enzyme may be further purified by redissolving in a salt solution such as a 5% solution of sodium chloride. The insoluble inactive material is filtered out and the filtrate dialyzed against distilled water or a dilute salt solution. The enzyme is again precipitated as described above. Further purification may be accomplished by means of column chromatography of a solution of the enzyme in a suitable solvent, using a suitable chromatographic medium, for example N,N'-diethylaminoethyl cellulose (DEAE cellulose). If desired, the enzyme in the dialyzate or eluate may be brought to a solid form

by lyophilization.

The galactose oxidase thus recovered is an extremely pure galactose oxidase having high activity for the oxidation of a galactose substrate and literally no activity with respect to glucose and other carbohydrates. Yields of galactose oxidase in amounts of about from 1,000 to 30,000 units per gram may be obtained with the procedures outlined above. This corresponds to galactose oxidase activity in the beer of about from 1 to 50 units per millilitre. The unit of galactose oxidase activity is defined as that weight in grams of galactose oxidase that will give the activity equivalent to one unit of glucose oxidase as defined by D. Scott, Jour. Agr. and Food Chem. *1*, 727 (1953).

The process above described has been found to produce commercially useful yields of galactose oxidase with fermentation times which are conveniently short and recovery methods which are adaptable to large scale production. Another advantage of this process is the fact that substrate costs are minimal.

The invention is further illustrated by the following Examples:

Example 1

A transfer from an agar slant stock culture of *Polyporus circinatus Fr.* into 25 ml. of Medium 1 in a 125 ml. Erlenmeyer flask was incubated as a still culture at room temperature for 2 days. The composition of Medium 1 is shown in Table 1 below:

TABLE 1 (Medium 1)

Ingredient	Concentration grams/ litre of tap water
Galactose	5.0
Potassium dihydrogen phosphate	7.5
Ammonium nitrate	1.0
Magnesium sulphate hydrate	0.5
*Trace element solution	1 ml.
*The trace element solution contains:	mg/100 ml.
Ferric nitrate hydrated	180
Sodium molybdate hydrate	85
Cuprous chloride	15
Manganous sulphate hydrate	65
Ferric sulphate hydrate	85

The pH of the final medium was adjusted to 6.3 and sterilized.

One litre of Medium 2 in a 2.8 litre Fernback flask was inoculated with 25 ml. of the still culture and incubated on a rotary shaker at room temperature until the fermentation beer assayed 5-20 units of galactose oxidase per ml. The time required was about 72 hours. The composition of Medium 2 is shown in Table 2 below:

TABLE 2 (Medium 2)

Ingredients	Concentration grams/ litre of tap water
Meat extract (Bactopeptone)	10
Glycine	5
Galactose	5
Potassium dihydrogen phosphate	7.5
Ammonium nitrate	1.0
Magnesium sulphate heptahydrate	0.25

The galactose oxidase was harvested as described above. The product was found to have an activity of 1,000-2,000 units of galactose oxidase per gram.

Satisfactory yields of galactose oxidase have been obtained by fermentations of from 72 to 120 hours of duration. If optimum enzyme wields are required it may be desirable to follow the course of fermentation by assaying the fermentation beer to determine the peak yield. One assay method which has been found useful for this purpose is an o-tolidine titration method for the assay of galactose oxidase. This assay is based upon the titration of hydrogen peroxide as it is formed during the oxidation of galactose by the enzyme galactose oxidase. The amount of o-tolidine oxidized by the hydrogen peroxide within the first 5 minutes after the addition of the enzyme to the substrate is a function of the enzyme activity and may be compared with standard curves which have previously been plotted using known concentrations of enzyme.

Other fermentations have been conducted utilizing various carbohydrate sources. The following example illustrates the use of several of these carbohydrates:

Example II

The fermentation was accomplished as in Example I. The yields obtained are shown in Table 3 below:

TABLE 3

Carbohydrate	Concentration grams/litre of medium	Yield units/ml.
Lactose	5	1 - 2
Glucose	5	8 - 10
Mannitol	5	8 - 10
Sorbitol	5	8 - 10
Inositol	5	8 - 10
Starch	5	8 - 10
Beet pulp	20	8 - 10
Glycerol	5	8 - 10
Sucrose	5	8 - 10

The protein or hydrolyzed protein may also be widely varied in carrying out the process of this invention. The following example illustrates the use of the various proteins and protein derivatives which have been found effective in carrying out the fermentation.

Example III

The procedure of Example I was followed except that various proteins and protein derivatives were used in place of Bactopeptone. These ingredients are shown in Table 4

below:

TABLE 4

Protein	Concentration grams/litre of medium	Yield units/ml.
Dry milk powder	10	8 - 10
Casein	10	5 - 10
Casein hydrolyzate	10	10 - 12
Trypticase	10	10 - 12
Meat extract solids	20	8 - 10
Soybean protein hydrolyzate	10	8 - 10
Autoclaved yeast	10	8 - 10
Fish solubles	30	1 - 2
Cotton-seed meal	20	5 - 10

Similarly, other auxiliary nitrogen sources than glycine have been utilized in carrying out the production of galactose oxidase according to the fermentation process of this invention. The following example illustrates the various auxiliary nitrogen sources:

Example IV

The fermentation was carried out as in Example I above except that various auxiliary nitrogen sources were used. These and the resulting yields obtained are shown in Table 5 below:

TABLE 5

Auxiliary Nitrogen Source	Concentration grams/litre of medium	Yield units/ml.
Ammonium acetate	5	8 - 10
Formamide	5	5 - 10
Glutamic acid	5	5 - 10
Aspartic acid	5	8 - 10
Asparagine	5	10 - 12
Alanine	5	10 - 12
Glycine	5	10 -12

The following Examples illustrate the process of the invention in which a lipid material is included in the fermentation material:

Example V

A transfer from an agar slant stock culture of *Polyporus circinatus Fr.* into 25 ml. of medium in a 125 ml. Erlenmeyer flask was incubated as a still culture at room temperature for 2 days. The composition of medium 1 is shown in Table 6 below:

TABLE 6 (Medium 1)

Ingredients	Concentration grams per litre
Galactose	5.0
Potassium dihydrogen phosphate	7.5
Ammonium nitrate	1.0
Magnesium sulphate hydrate	0.5
*Trace element solution	1 ml.
*The trace element solution contains:	mg/100 ml.
Ferric nitrate, hydrated	180
Sodium molybdate hydrate	85
Cuprous chloride	15
Manganous sulphate hydrate	65
Ferric sulphate hydrate	85

The pH of the final medium is adjusted to 6.3 and sterilized.

One litre of Medium 2 in a 2.8 litre Fernback flask was inoculated with 25 ml. of the still culture and incubated on a rotary shaker at room temperature until the fermentation beer assayed 5-20 units of galactose oxidase per ml. The time required was about 72 hours. The composition of Medium 2 is shown in Table 7 below:

TABLE 7 (Medium 2)

Ingredients	Concentration grams per litre
Galactose	5
Casein Hydrolyzate	10
Glycine	5
Lard Oil (Larex EWS)	10
Octadecanol	1
Potassium dihydrogen phosphate	0.15
Ammonium nitrate	1.0
Magnesium sulphate heptahydrate	0.12
Vitamin mixture	(1 tablet)

For the vitamin mixture any readily soluble multiple vitamin composition may be used. Such composition usually contains:

Vitamin A	5000 U.S.P. Units
Vitamin D	500 U.S.P. Uhits
Vitamin C	50 mg.
Vitamin B_1	2 mg.
Vitamin B_2	2.5 mg.
Vitamin B_6	1 mg.
Niacinamide	20 mg.
Vitamin B_{12}	1 mcg.

This medium was pH adjusted to pH 6.8 with ammonium hydroxide and sterilized.

The galactose oxidase was harvested as described above. The product was found to have an activity of 1,000-2,000 units of galactose oxidase per gram.

Example VI

The fermentation was accomplished as in Example V. The yields obtained are shown in Table 8 below:

TABLE 8

Carbohydrate	Concentration grams per litre	Yield units per millilitre
Galactose	5	30 - 50
Lactose	5	3 - 4
Glucose	5	20 - 40
Mannitol	5	10 - 30
Sorbitol	5	10 - 30
Inositol	5	10 - 30
Beet pulp	20	30 - 50
Glycerol	5	10 - 12
Sucrose	5	20 - 40

The protein or hydrolyzed protein may also be widely varied in carrying out the process of this invention. The following example illustrates the use of various proteins and protein derivatives which have been found effective in carrying out the fermentation.

Example VII

The procedure of Example V was followed except that various proteins and protein derivatives were used in place of casein hydrolyzate. These ingredients are shown in Table 9 below:

TABLE 9

Protein	Concentration grams per litre	Yield units per millilitre
Dry milk powder	10	10 - 30
Casein	10	20 - 50
Meat extract (Bactopeptone)	10	20 - 50
Trypticase	10	20 - 50
Meat extract solids	20	20 - 50
Soybean protein hydrolyzate	10	10 - 15
Autoclaved yeast	10	20 - 40
Fish solubles	20	5 - 10

Similarly, other auxiliary nitrogen sources than glycine have been utilised in carrying out the production of galactose oxidase according to the fermentation process of this invention. The following examples illustrates the use of various auxiliary nitrogen sources.

Example VIII

The fermentation was carried out as in Example V above except that various auxiliary nitrogen sources were used. These and the resulting yields obtained are shown in Table 10 below:

TABLE 10

Auxiliary Nitrogen Source	Concentration grams per litre	Yield units per millilitre
Ammonium acetate	5	15 - 30
Formamide	5	5 - 10
Glutamic acid	5	5 - 10
Aspartic acid	5	8 - 10
Asparagin	5	10 - 20
Alanine	5	15 - 30
Glycine	5	30 - 50

Various lipid materials have been found to be satisfactory for use in the processes of this invention. The following example illustrates the use of other lipid materials.

Example IX

Following the procedure of Example V above, fermentations were carried out using other lipid materials in place of lard oil and

octadecanol. The various lipid materials and the resulting yields are shown in Table 11 below:

TABLE 11

Lipid Material	Concentration grams per litre	Yield units per millilitre
Octadecanol	10	5 - 10
Octadecanol + Lard oil (Larex EWS)	0.2 10	30 - 50
Stearic acid	10	15 - 30
Mutton tallow	10	30 - 50
Cotton-seed oil	10	20 - 40
Olive oil	10	20 - 40
Linseed oil	10	20 - 40

WHAT WE CLAIM IS:

1. A process for the production of galactose oxidase which comprises growing a culture of *Polyporus circinatus Fr.* in an aqueous fermentation medium containing up to 2.0% (w/v) of a carbohydrate source comprising one or more of galactose, lactose, sucrose, raffinose, glucose, fructose, mannose, sorbose, beet pulp, orange pulp, flour, starch, mannitol, sorbitol, inositol and glycerol, from 0.01% to 0.15% (w/v) of nitrogen in the form of a primary organic nitrogen source comprising one or more of casein, casein hydrolysate, whole milk powder, meat extracts, fish solubles, autoclaved yeast, soybean protein hydrolysate, cottonseed meal and stick liquors, and from 0.005% to 0.1% (w/v) of nitrogen in the form of an auxiliary nitrogen source comprising one or more of glycine, aspartic acid, asparagine, glutamic acid, alanine, ammonium acetate, formamide and urea.

2. A process as claimed in Claim 1 wherein the fermentation medium also contains from 0.5% to 2.0% (w/v) of a lipid material comprising one or more of mutton tallow, lard oil, linseed oil, peanut oil, olive oil, soybean oil, vegetable oil, corn oil, cottonseed oil, octadecanol, stearic acid and sorbitan trioleate.

3. A process as claimed in claim 1 or 2 wherein the culture of *Polyporus circinatus Fr.* is grown at a temperature from 23°C. to 30°C. and at an initial pH of 6.3 to 6.8 for a period of 72 to 120 hours.

4. A process as claimed in claim 1 substantially as described with reference to any of the Examples.

5. Galactose oxidase when produced by the process claimed in any of the preceding claims.

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